



Review

In vitro human digestion models for food applicationsSun Jin Hur^{a,*}, Beong Ou Lim^b, Eric A. Decker^c, D. Julian McClements^c^a Institute of Agriculture & Life Science, Gyeongsang National University, 900 Kajwa-dong, Jinju, Gyeongnam 660-701, Republic of Korea^b Department of Applied Biochemistry, Konkuk University, 322 Danwol-dong, Chungju, Chungbuk 380-701, Republic of Korea^c Department of Food Science, University of Massachusetts Amherst, 100 Holdsworth Way, Amherst, MA 01003, USA

ARTICLE INFO

Article history:

Received 27 December 2009

Received in revised form 21 June 2010

Accepted 16 August 2010

Keywords:

In vitro digestion models

Enzymes

Gastrointestinal tract

Foods

ABSTRACT

In vitro digestion models are widely used to study the structural changes, digestibility, and release of food components under simulated gastrointestinal conditions. However, the results of *in vitro* digestion models are often different to those found using *in vivo* models because of the difficulties in accurately simulating the highly complex physicochemical and physiological events occurring in animal and human digestive tracts. This paper provides an overview of current trends in the development and utilisation of *in vitro* digestion models for foods, as well as information that can be used to develop improved digestion models. Our survey of *in vitro* digestion models found that the most predominant food samples tested were plants, meats, fish, dairy, and emulsion-based foods. The most frequently used biological molecules included in the digestion models were digestive enzymes (pancreatin, pepsin, trypsin, chymotrypsin, peptidase, α -amylase, and lipase), bile salts, and mucin. In all the *in vitro* digestion models surveyed, the digestion temperature was 37 °C although varying types and concentrations of enzymes were utilised. With regard to digestion times, 2 h (the stomach, small intestine, and large intestine each) was predominantly employed. This survey enhances the understanding of *in vitro* digestion models and provides indications for the development of improved *in vitro* digestion models for foods or pharmaceuticals.

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1. Introduction

In the past few years, there has been an increasing interest in the structural design of food-based delivery systems to encapsu-

late, protect, and release bioactive components believed to benefit human health (McClements, Decker, & Park, 2009). These delivery systems may be designed to release the bioactive components at a specific location in the human gastrointestinal (GI) tract, often in response to an environmental trigger, such as pH, ionic strength, or enzyme activity. The delivery system may also be designed to control the rate of bioactive release, such as burst release or prolonged release. Testing the efficacy of newly developed delivery

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systems depends on the availability of digestion models that accurately simulate the complex physicochemical and physiological events that occur in the human GI tract.

In vivo feeding methods, using animals or humans, usually provide the most accurate results, but they are time consuming and costly, which is why much effort has been devoted to the development of *in vitro* procedures (Boisen & Eggum, 1991). In principle, *in vitro* digestion models provide a useful alternative to animal and human models by rapidly screening food ingredients. The ideal *in vitro* digestion method would provide accurate results in a short time (Coles, Moughan, & Darragh, 2005) and could thus serve as a tool for rapid screening foods or delivery systems with different compositions and structures. In practice, any *in vitro* method is inevitably going to fail to match the accuracy that can be achieved by actually studying a food *in vivo* due to the inherent complexity of the process (Coles et al., 2005; Fuller, 1991). Consequently, some compromise is needed between accuracy and ease of utilisation of any *in vitro* digestion model. During the past few years, food and animal scientists have utilised a number of *in vitro* digestion models to test the structural and chemical changes that occur in different foods under simulated GI conditions, although none of these methods has yet been widely accepted. The purpose of this paper is to provide an overview of the current status of *in vitro* digestion models, and to provide information that can be used as the basis for the development of standardised digestion models.

2. Summary of survey

We surveyed more than 80 studies conducted in the past 10 years that were related to *in vitro* digestion models for foods (Table 1). There were important differences in these studies, which depended on the specific food component being analysed, the nature of the food matrix, and the sophistication of the *in vitro* digestion model used. The survey found that the most predominant food samples tested using *in vitro* digestion models were: plant-based foods, such as starch, tea, rice, or bread (45%); meats (18%); dairy foods (9%); marine foods (9%); and, emulsions (9%). The *in vitro* digestion models surveyed also differed from one another in their operation:

- (1) The number and type of steps included in the digestion sequence, e.g., mouth, stomach, small intestine, large intestine.
- (2) The composition of the digestive fluids used in each step, e.g., enzymes, salts, buffers, biological polymers, and surface-active components.
- (3) The mechanical stresses and fluid flows utilised in each step in the digestion sequence, e.g., magnitude and direction of applied stresses, flow geometries, and flow profiles.

In addition, there are considerable differences in the type of experimental parameters measured in the various digestion models. These include chemical changes (such as hydrolysis of lipids, proteins, and/or polysaccharides), location changes (such as release of encapsulated components, competitive adsorption processes, multilayer formation), and structural changes (such as breakdown of specific structures, aggregation, droplet coalescence, or droplet disruption).

The most frequently utilised enzymes and other biological molecules used within *in vitro* digestion models were pepsin, pancreatin, trypsin, chymotrypsin, peptidase, α -amylase, lipase, bile salt, and mucin. Several studies have utilised enzymes collected from human subjects, whereas others have used enzymes extracted from animal or plant sources. For instance, Almaas et al. (2006) studied gastric juice and duodenal juice collected from human subjects. Chattertona, Rasmussen, Heegaard, Sorensenb, and Petersenb

(2004) utilised gastric juice collected from human infants. Kitabatake and Kinekawa (1998) studied mixed enzymes, e.g., porcine pepsin, human pepsin, rat gastric juice, and rat pancreatic juice. It should be noted that most enzymes utilised for *in vitro* digestion studies are collected or extracted from omnivorous animals, i.e., pigs, rats, or human volunteers.

The types of enzyme included within an *in vitro* digestion model tend to reflect the major food components being investigated, e.g., lipases for lipid digestion, proteases for protein digestion, and amylases for starch digestion. For example, in their studies of lipid digestion in oil-in-water emulsions, Mun, Decker, and McClements (2007) and Porter et al. (2004) utilised only pancreatic lipase or pancreatin (which contains pancreatic lipase), respectively. Studies of the digestion of more complex multi-component food systems tend to utilise a wider range of digestive enzymes, such as α -amylase, mucin, pepsin, pancreatin, and lipase (Naim, Messier, Saucier, & Piette, 2004; Savage & Catherwood, 2007; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005; Xing, Yang, Chan, Tao, & Wong, 2008; Hur, Decker, & McClements, 2009a; Brandon et al., 2006) have. It should be noted that different enzymes are usually added sequentially, rather than all together, so as to simulate the different steps of the digestive process. For example, many *in vitro* models are based on consecutive incubations with pepsin to simulate the stomach and then pancreatin to simulate the small intestine (Boisen & Eggum, 1991). The enzyme composition of a particular digestive fluid can often be simulated by mixing together appropriate amounts of pure enzymes (Boisen & Eggum, 1991). It should also be noted that enzymes often require additional components within the digestive fluids to operate efficiently, e.g., pancreatic lipase requires the presence of calcium and bile salts (Boisen & Eggum, 1991). Finally, it should be noted that the activity of an enzyme preparation may decrease over time, and so it is important to prepare them freshly for each study.

For all *in vitro* digestion models surveyed in this review, the digestion temperature was 37 °C despite the variations in the enzymes employed. The length of the incubation times of samples in the various simulated digestive fluids should mimic the reported digestion times in humans. In practice, a range of digestion times has been reported for incubation of test samples in simulated stomach, small intestine, and large intestine fluids (Table 1). An important factor influencing the digestion time is the nature of the sample being tested. It is known that large food particles move through the stomach more slowly than small ones, since they have to be small enough (<1 mm) to pass through the pylorus valve separating the stomach and small intestine. A food containing large particles therefore requires a longer incubation time in the stomach. Thus, the most relevant digestion time must be considered when designing *in vitro* digestion models for testing foods containing relatively large particles. The concentration and composition of enzymes are also very important factors to consider when designing *in vitro* digestion models. Typically, higher enzyme concentrations accelerate digestion or degradation of food components, and so it is important to use physiologically relevant levels. These levels depend on the person involved, their mental state, age and health status, the time of day the food is consumed, and the type and amount of food consumed. The *in vitro* digestion models surveyed used a variety of different enzyme levels (Table 1), which will lead to variations in the results between studies. The most common parameters measured in the surveyed *in vitro* digestion studies were: digestibility/degradation > bioaccessibility > sample stability > structural changes.

2.1. Cell culture models

Cell culture models have also been utilised as part of *in vitro* digestion models. In particular, the Caco-2 cell culture model has

Table 1Survey of *in vitro* digestion systems used to test various foods.

Plant-based foods				
Samples	Measurement parameters	Enzymes or chemicals	Digestion times	Literature references
Pectin	Allergen, gel-forming	Pepsin in HCl pancrease	15 min 1 h and 15 h	Polovic et al. (2008)
Bioaccessibility of mycotoxins in infant formula	Amount of mycotoxins and bioaccessibility	α -Amylase, mucin BSA, pepsin pancreatin, lipase, bile salt	5 min 2 h 2 h	Versantvoort et al. (2005)
Digestion recovery of green tea catechins	Catechin profiles, catechin contents, catechin recovery	Pepsin lipase pancreatin bile	1 h 2 h	Green et al. (2007)
Degradation of Phytate	Phytate contents, phytate degradation, phytate solubility	Pepsin pancreatin bile salt	10 min or 20 min 30 min or 1 h	Haraldsson, Veide, Andlid, Alminger, and Sandberg (2005)
Determination of oxalates in Japanese Taro corms	Dry matter, gastric and intestinal soluble oxalate, total oxalate	α -Amylase, mucin BSA, pepsin pancreatin, lipase, bile salt	5 min 2 h 2 h	Savage and Catherwood (2007)
Iron uptake and transport from rye bread	Iron uptake, iron dialysate concentration, iron absorption	Pepsin pancreatin bile salt	1 h	Bering et al. (2006)
Digestion of grape lipid transfer protein (LTP)	Digestion of grape LTP, biologic activity of LTP, allergen concentration	Pepsin bile salt trypsin chymotrypsin	2 h 2 h	Vassilopoulou et al. (2006)
Antioxidant capacity of green tea in meat (with iron, ascorbate and casein)	Antioxidant capacity, polyphenol contents, ferrous iron contents	Pepsin pancreatin bile salt	2 h 2 h	Alexandropoulou, Komaitis, and Kapsokafalou (2006)
Starch digestibility	Hydrolysis, kinetics of starch digestion	α -Amylase	0–180 min	Bravo et al. (1998)
Spelt protein digestion	Protein contents	Trypsin chymotrypsin peptidase pepsin pancreatin	30 min 6 h	Abdel-Aal (2008)
Starch digestion	Digestion rate of starch, concentration of starch	Pepsin α -amylase amylglucosidase	0–15 h	Wolf, Bauer, and Fahey (1999)
Allergenicity of kiwi allergens	Digestion of allergens	Pepsin bile salt pancreatic lipase co-lipase trypsin chymotrypsin	0–120 min	Bublin et al. (2008)
Enzymatic digestion of oat fractions by lactobacilli	Growth of lactobacilli	α -amylase pepsin pancreatin	30 min 1 h 1 h	Kedia, Vazquez, and Pandiella (2008)
Bioaccessibility of wheatgrass	Bioaccessible concentrations of wheatgrass	Pepsin pancreatin bile salt	3 h 4 h	Kulkarni, Acharya, Rajurkar, and Reddy (2007)
Digestive of grape seed flavonoids	Stability and recovery of phenolic compound, activity of Caco-2 cell hydrolases	α -amylase pepsin pancreatin bile salt	10 min 1 h 2 h	Laurent, Besancon, and Caporiccio (2007)
Impact of triglycerides on bioaccessibility of dietary carotenoids	Micellarization of carotenes, carotenoid uptake efficiency	Pepsin lipase pancreatin bile salt	1 h 2 h	Huo, Gerruzzi, Schwartz, and Failla (2007)
Carotenoid bioavailability from baby meals	% Carotenoids in micelles, micellarization of carotenoids	Pepsin pancreatin	1 h 2 h	Garrett, Failla, and Sarama (1999)

(continued on next page)

Table 1 (continued)

Plant-based foods				
Samples	Measurement parameters	Enzymes or chemicals	Digestion times	Literature references
Digestibility of soya bean, cowpea and maize	pH change, Lipid content, Solubility, absorbability and digestibility	bile salt α -amylase lipase pepsin pancreatin	30 min 1 h 30 min	Kiers, Nout, and Rombouts (2000)
Digestibility of soy lunasin and Bowman birk protease inhibitor (BBI)	Digestibility of lunasin and BBI, variation and internalisation of soy lunasin and BBI	Pepsin pancreatin	0–120 min 0–120 min	Park, Jeong, and Lumen (2007)
Digestion of phenolic compounds, glucosinolates and Vitamin C	Flavonoid stability of broccoli, glucosinolate and vitamin C stability	Pepsin pancreatin bile salt	2 h 2.5 h	Vallejo, Gil-Izquierdo, Pearez-Vicente, and Garciaa-Viguera (2004)
Digestion of starch	Digestion coefficients and characteristics, digestion of horsebeans	Pepsin enzyme cocktail (pancreatin and amyloglucosidase)	30 min 0–6 h	Weurding, Veldman, Veen, van der Aar, and Verstege (2001)
Orange juice flavonone solubility and transformation to chalcones	Total flavanones and chalcones	Pepsin pancreatin bile salt	2 h 2.5 h	Gil-Izquierdo, Gil, Tomaas-Barberaan, and Ferreres (2003)
Efficiency of beta carotene micellarisation	pH change, beta carotene transfer	Pancreatin bile salt	2 h	Wright, Pietrangelo, and MacNaughton (2008)
<i>In vitro</i> availability of iron and zinc in pearl millet flours	<i>In vitro</i> available iron and zinc contents, iron and zinc <i>in vitro</i> availability	Pepsin pancreatin bile salt	1 h 2 h	Lestienne, Besancon, Caporiccio, Lullien-Peallerin, and Treache (2005)
Effect of phytase on phytate degradation	Phatase stability, phatate degradation, mineral solubilisation	Pepsin pancreatin	0–1 h 44 min 0–1 h 44 min	Pontoppidan, Pettersson, and Sandberg (2007)
α -Amylase digestion of starches	Transmission electron microscopy, size-exclusion chromatograms	Pancreatic amylase	2 h	Evans & Thompson (2004)
Pepsin digestion of sorghum	Sorghum digestibility, sorghum protein digestibility	Pepsin	0–120 min	Nunes et al. (2004)
Stability of polyphenols in chokeberry	Recovered phenolics, stability of polyphenols	Pepsin pancreatin bile salt	2 h 2 h	Bermúdez-Soto et al. (2007)
Digestion of rice dough in the presence of alginate	Scanning electron microscopy, Fourier transform infrared spectroscopy	α -amylase	3 h	Koh et al. (2009)
Degradation of barley β -glucan in wheat bread	Scanning electron micrographs, distribution of β -glucan	Pepsin α -amylase	30 min 5 h	Cleary, Andersson, and Brennan (2007)
Bioactivity of wheat bread	Total phenolics, flavonoids, phenolic acids content, lipid peroxidation inhibition	α -amylase pepsin pancreatin bile salt	10 min 2 h 1 h	Gawlik-Dziki, Dziki, Baraniak, and Lin (2009)
<i>In vitro</i> digestion of starch	Percent digestion of starch	α -amylase	0–24 h	Zhang et al. (1996)
Develop a model stomach system and to investigate the kinetics of food disintegration	Food disintegration and stomach emptying, disintegration and texture change, kinetic parameters	α -amylase mucin pepsin mucin	30sec 2 h	Kong and Singh (2008)
Angiotensin I-converting enzyme (ACE) inhibitory activity of soy protein digests	Degree of hydrolysis, ACE-inhibitory activity assay	Pepsin pancreatin	1 h 2 h	Lo and Li-Chan (2005)
Availability of various iron fortificants in bread and milk	Iron availabilities and amount of dialysed iron from breads and milk	Pepsin pancreatin bile salt	1 h 2 h	Yeung, Glahn, & Miller (2002)
Digestible iron and zinc content of Okra sauce	Composition of Okra sauce, iron and zinc content	Pepsin pancreatin bile salt	1 h 2 h	Avallone, Bohuon, Hemery, and Theche (2007)
Determination of ascorbic acid:Fe in rice cereal	Iron and Fe availability	Pepsin pancreatin bile salt	1 h 2 h	Glahn, Lee, and Miller (1999)

Table 1 (continued)

Plant-based foods				
Samples	Measurement parameters	Enzymes or chemicals	Digestion times	Literature references
Iron bioavailability from raisin containing foods	Iron bioavailability	Pepsin pancreatin bile salt	1 h 2 h	Yeung et al. (2003)
Phenolic compounds in fruits	Antioxidant activity, total polyphenol, profile of polyphenols	Pepsin pancreatin	2 h 4 h	Tarko, Duda-Chodak, Sroka, Satora, and Michalik (2009)
Phenolic compounds in sweet cherries		Pepsin pancreatin bile salt	2 h 2 h	Fazzari et al. (2008)
Meat-related foods				
Samples	Measurement parameters	Enzymes or chemicals	Digestion times	Literature references
Lamb meat myofibrillar protein oxidation	Myofibrillar protein digestibility	Pepsin mucosa trypsin α -chymotrypsin	1 h 0,10,20,30,40,60 min, 0,5,10,20,30 min	Santé-Lhoutellier, Engel, Aubry, & Gatellier (2008)
Digestion of sarcoplasmic and myofibrillar protein	Nitrogen and iron solubility and molecular weight distribution, recovery of ^{59}Fe , amino acid composition	Pepsin pepsin/pancreatin	2 h 2 h	Storcksdieck, Bonsmann, and Hurrell (2007)
Effect of iron and red wine on antioxidant capacity in meat	Antioxidant capacity, phenolic and iron content	Pepsin pancreatin bile salt	2 h 2 h	Argyri, Komaitis, and Kapsokafalou (2006)
Screening of microbe in Iberian sausage	Characterisation of the potential probiotic isolates	Oxgall pancreatin	0–8 h 3 h	Ruiz-Moyano, Martin, Benito, Nevado, and Cordoba (2008)
Effect of cooking on iron and bioactive compounds	Iron concentration and bioactive compounds	Pepsin pancreatin bile salt	2 h 1 h	Purchas, Busboom, and Wilkinson (2006)
Digestion of meat protein	Profile of peptides, proteolysis	Pepsin trypsin chymotrypsin	120 min 130 min	Gatellier & Sante-Lhoutellier (2009)
Digestion of myofibrillar protein in lamb meat	Carbonyl content, proteolytic activity, digestibility of myofibrillar protein	Pepsin trypsin α -chymotrypsin	0–60 min 0–30 min	Santé-Lhoutellier et al. (2008)
Non-haem iron availability from pork	Iron availability, iron-reducing capacity, molecular weight	Pepsin pepsin + pancreatin	1 h 1 h	Sørensen, Sørensen, Søndergaard, and Bukhave (2007)
Bioaccessibility of heterocyclic amines from cooked meat	Release of heterocyclic amines	Amylase pepsin pancreatin	10 min 30 min 3.5 h	Kulp, Fortson, Knize, and Felton (2003)
Evaluate survival of E-coli O157:H7 in sausage	Survival or growth of E-Coli O157:H7 cells,	α -amylase lysozyme mucin pepsin pancreatin bile salt	1 min 2 h 4 h	Naim et al. (2004)
Digestion of animal byproduct proteins	Chemical composition and protein evaluation	Pepsin pancreatin	1 h 24 h	Howie, Calsamiglia, and Stem (1996)
Concentration of bioavailable iron and development of dialyzability method	pH, dialyzability	Pepsin pancreatin bile salt	2 h 2 h	Argyri, Birba, Miller, Komaitis, and Kapsokafalou (2009)
Dialysable iron in chicken proteins	Production of dialyzable iron	Pepsin pancreatin bile salt	2 h 2 h	Diaz, Vatter, and Mahoney (2002)
<i>In vitro</i> digestion of different meat sources	Iron and nitrogen solubility and molecular weight distribution, amino acid composition	Pepsin pepsin/pancreatin		Storcksdieck et al. (2007)
<i>In vitro</i> digestion of beef with various fibres	Confocal microscopy, free fatty acid contents fatty acid composition cholesterol contents	α -amylase mucin BSA pepsin mucin pancreatin lipase bile salt	5 min 2 h 2 h	Hur, Lim, Decker, and McClements (2009)

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Table 1 (continued)

Dairy foods				
Samples	Measurement parameters	Enzymes or chemicals	Digestion times	Literature references
Digestibility of milk whey protein	Digestion of whey protein, size exclusion chromatography patterns	Porcine pepsin human pepsin chymosin pancreatin rat gastric juice rat pancreatic juice	1 h 1 h 1 h 1 h 1 h 1 h	Kitabatake and Kinekawa (1998)
Enzyme inhibitory activity from pea and whey protein	Angiotensin I-converting enzyme inhibitory activity, soluble fraction of non-digested pea and whey protein	Pepsin trypsin α -amylase	2 h 2.5 h	Vermeirssen, Camp, and Verstraete (2005)
Digestion of bovine and caprine milk	pH change, degradation of protein, digestibility of milk	Human gastric juice human duodenal juice	0–30 min 0–30 min	Almaas et al. (2006)
Digestion of milk protein ingredients	pH change Digestion of milk and peptide	Porcine pepsin infant gastric juice	1 h 1 h	Chatterton et al. (2004)
Digestion of peptides in emmental cheese	Total nitrogen, peptide hydrolysis, angiotensin-I converting enzyme	Pepsin trypsin pancreatin	30 min 4 h	Parrot, Degraeve, Curia, and Martial-Gros (2003)
Characterisation of β -lactoglobulin peptide	Determination of digestion of β -lactoglobulin by mass spectrometric techniques	Pepsin bile salt trypsin α -chymotrypsin	2 h 1 h	Moreno, Quintanilla-Lopez, Lebron-Aguilar, Olano, and Sanz (2008)
Producibility and digestibility of antihypertensive tripeptides	Residual ratio of antihypertensive tripeptides	Pepsin pancreatin trypsin chymotrypsin	2 h 2 h	Ohsawa et al. (2008)
Marine foods				
Samples	Measurement parameters	Enzymes or chemicals	Digestion times	Literature references
Bioaccessibility of polychlorinated biphenyls (PCBs) in fish and vegetables	Concentrations of PCBs, bioaccessibility of PCBs	Pepsin pancreatin lipase bile extract porcine α -amylase	1 h 6 h 6 h 6 h 6 h	Xing et al. (2008)
Antioxidative property of herring press juice	Protein concentration, electrophoretic pattern of digestion, oxygen radical absorbance capacity	Pepsin pancreatin bile extract		Sannaveerappa, Westlund, Sandberg, and Undeland (2007)
Enzyme inhibitory peptides from bonito meat	Angiotensin I-converting enzyme inhibitory activity, peptide concentration and sequencing	Trypsin chymotrypsin	2–12 h	Hasan, Kitagawa et al. (2006)
Production kinetics of peptides from bonito meat pepsin	Angiotensin-I-converting enzyme inhibitory activity, production of inhibitory peptides	Pepsin	0–50 h	Hasan, Kitagawa et al. (2006)
Selenium and mercury bioaccessibility in fish	Bioaccessibility of selenium, selenium and mercury concentration	Pancreatin amylase bile salt	4 h 4 h	Cabañero, Madrid, and Cámara (2004)
Digestion of seaweeds protein	Digestion of protein	Pepsin pancreatin	30 min 24h	Goñi, Gudiel-Urbano, and Saura-Calixto (2002)
Selenium bioaccessibility assessment	Selenium content, characterisation of selenium	Pepsin pancreatin α -amylase bile salt	4 h 4 h	Reyes, Encinar, Marchante-Gayón, Alonso, and Sanz-Medel (2006)

Table 1 (continued)

Emulsion-based foods				
Samples	Measurement parameters	Enzymes or chemicals	Digestion times	Literature references
Digestion of emulsified lipid	Optical microscopy, ζ -potential, particle diameters, particle size distribution	Pancreatic lipase	2 h	Mun et al. (2007)
Self emulsifying emulsion® (drug)	Microscopical images, droplet size	Bile salt pancreatin,		Abdalla, Klein, and Mäder (2008)
Digestion of emulsified corn oil	Optical microscopy, ζ -potential, particle diameters free fatty acid release	Pancreatin bile salt	2–24 h	Sandra, Decker, and McClements (2008)
Digestion of emulsified lipids	Optical & confocal microscopy, ζ -potential, particle size, creaming stability,	α -amylase mucin BSA pepsin mucin pancreatin lipase bile salt	5 min 2 h 2 h	Hur et al. (2009b)
Influence of dietary fibre on emulsion	Creaming stability, ζ -potential, optical microscopy, particle size,	Pancreatin bile salt	2 h	Beysseriat, Decker, and McClements (2006)
Mineral, iron and lipids-related foods				
Samples	Measurement parameters	Enzymes or chemicals	Digestion times	Literature references
Iron bioavailability	Cell ferritin formation	Pepsin pancreatin bile salt	1 h 2 h	Mahler et al. (2009)
Mineral bioavailability of calcium carbonate tablets and powder milk	Profile of dialysed calcium, bioavailability of calcium, recovery of calcium, concentration of sodium bicarbonate dialysing solution	Pepsin pancreatin bile salt	2 h 2 h	Shiowatana, Kitthikhun, Sottimai, Promchan, and Kunajiraporn (2006)
Gastric pH and mineral dialysability	Percent iron dialyzability	Pepsin pancreatin bile salt	2 h or 30 min 2 h or 1 h	Drago, Binaghi, and Valencia (2005)
Triglycerides	Lipid concentration	Pancreatin	30 min	Sek, Porter, and Charman (2001)
Bioaccessibility of contaminant and its risk assessment	Lead, phthalates, azo dyes and benzoic acid contents.	α -amylase, mucin BSA, pepsin pancreatin, lipase, bile salt	5 min or 30 min 2 h 2 h	Brandon et al. (2006)
Lipid-based drug delivery system	Rate of lipolysis, ζ -potential, cryogenic transmission electron microscopy	Pancreatin bile salt	0–30 min	Fatouros et al. (2007)
Triglyceride-based poorly water-soluble drugs	Pharmacokinetic Parameters	Pancreatin	30 min, 1 h	Porter et al. (2004)
Lipophilic drug	Amount of dexamethasone and griseofulvin Pharmacokinetic	Pancreatin	30– 90 min	Dahan and Hoffman (2007)

The digestion times reflect the length of time that the sample was incubated in the presence of the indicated digestive enzymes or chemicals.

been widely used as a predictive tool for the absorption of bioactive components from foods and pharmaceutical preparations. The *in vitro* digestion/Caco-2 cell culture model developed by Glahn et al. (1998) offers a rapid, low-cost method for screening foods and food combinations for iron bioavailability before more definitive human trials (Mahler, Shuler, & Glahn, 2009). In the present review, most Caco-2 cell model studies were noted to be iron uptake-related studies, and Mahler et al. (2009) reported that the estimation of iron bioavailability from the *in vitro* digestion/Caco-2 cell culture model has been well correlated, qualitatively, with human data. The results from quantitative studies comparing hu-

man *in vivo* to *in vitro* Caco-2 iron uptake results for semi-synthetic meals have shown that human and Caco-2 data generally agree (Mahler et al., 2009).

3. *In vitro* digestion and enzymes

Several factors, such as sample characteristics, enzyme activity, ionic composition, applied mechanical stresses and digestion times, have significant influences on the results of *in vitro* digestion methods. Therefore, *in vivo* conditions can never be completely simulated under *in vitro* conditions (Boisen & Eggum, 1991). An

early study by Boisen and Eggum (1991) defined the relationship between *in vitro* digestion and enzyme activity. They reported that the *in vitro* technique can be designed to use specific enzymes either to give maximal digestibility values or to measure the initial rate of hydrolysis. The most important factor in an *in vitro* digestion system is the enzyme characteristics. Several factors, such as concentration, temperature, pH, stability, activators, inhibitors, and incubation time, affect enzyme activities (Boisen & Eggum, 1991). The choice of enzymes and incubation conditions and the need for equipment are also dependent on the objectives of the study (Boisen & Eggum, 1991). Single-enzyme methods can be useful for predicting the digestibility of single nutrients, e.g., protein by the use of pepsin, starch by the use of amylase, or lipids by the use of lipases (Boisen & Eggum, 1991).

It has been reported that using a single purified enzyme, rather than a complex biological mixture, is often advantageous because it facilitates the standardization of *in vitro* digestion models, which enables more consistent laboratory-to-laboratory comparisons (Coles et al., 2005). However, the digestion of one nutrient is often influenced by the digestion of other nutrients, and so it is often more realistic to use a complex mixture of enzymes rather than a single purified one (Boisen & Eggum, 1991).

3.1. Lipases

Lipases are present in the stomach (gastric lipase) and pancreas (pancreatic lipase), where they absorb to the surfaces of emulsified lipids and convert triacylglycerols and diacylglycerols to monoacylglycerols and free fatty acids. These lipid digestion products are these solubilised within mixed micelles and vesicles that transport them to the epithelium cells through the mucous layer. The activity of pancreatic lipase depends on the presence of co-lipase, bile salts, and calcium (Kimura, Futami, Tarui, & Shinomiya, 1982; Zangenberg, Müllertz, Kristensen, & Hovgaard, 2001). Pancreatic lipase has an absolute requirement for Ca^{2+} , which binds in a stoichiometric ratio of 1:1 to the lipid substrate and the enzyme (Carey, Small, & Bliss, 1983). Fatouros and Mullertz, (2008) reported that calcium reacts with liberated free fatty acids by means of ionic complexation, thereby removing them from the surface of the lipid droplets and preventing them from inhibiting the lipase. They also reported that when calcium is added at the start of the lipolysis, it results in a very fast initial lipolysis rate followed by a leveling out at longer times, which was attributed to product inhibition by free fatty acids and possibly precipitation of bile salts with calcium. It has therefore been proposed that it is better to add calcium continuously throughout the *in vitro* digestion process, rather than adding it all at the beginning. The bile salts and phospholipids are surfaced active molecules that adsorb to droplet surfaces and displace any existing emulsifier molecules. This change in interfacial composition can facilitate the subsequent adsorption of the lipase-co-lipase complex to the lipid droplet surfaces. Bile salts and phospholipids also form mixed micelles and vesicles in the aqueous phase, which are capable of incorporating lipid digestion products and removing them from the lipid droplet surfaces. It is therefore important to include the appropriate amounts of lipase, co-lipase, bile salts, phospholipids and calcium in an *in vitro* digestion model for lipid digestion.

As a response to the intake of a meal, bile is secreted into the duodenum, and in the fed state (30 min after intake of a meal), the mean bile salt concentrations in human duodenal (Lind, Jacobsen, Holm, & Müllertz, 2007) and jejunal fluids are between 8 mM (Persson et al., 2006) and 12 mM (Kalantzi et al., 2006). Zangenberg et al. (2001) reported that the mean values of bile salt were typically between 5 and 15 mM, depending on the time after ingestion of the meal, with peak values up to 40 mM. The lipid hydrolysis rate is influenced by bile salt and lipase concentrations. Patton

(1981) and Zangenberg et al. (2001) suggested that the activity of $1\text{--}2 \times 10^{-7}$ M pancreatic lipase and co-lipase in intestinal fluids was between 150 and 300 units/ml. In order to ensure lipase activity in excess, an interval of 270–1340 units/ml was investigated (Zangenberg et al., 2001).

Lipases are perhaps the most frequently used enzymes in organic chemistry. Segura et al. (2004) reported that most commercial preparations of lipases are crude preparations that may contain other proteins, which in many cases can have opposite activities and make the understanding of the results difficult. They also found that the three main components of porcine pancreatic lipase extract present very different catalytic properties: the enantioselectivity was very different, as well as the influence of the reaction conditions.

3.2. Proteases

Proteases are mainly present in the stomach (pepsin) and small intestine (trypsin and chymotrypsin) where they are responsible for breaking down proteins/peptides into smaller peptides and amino acids. The daily pepsin secretion in adults is 20–30 kU of enzyme activity at 37 °C, equivalent to around 10 mg, while a typical adult dietary intake of protein comprises around 75 g/24 h, giving a pepsin/protein ratio of 1:7500 (Bublin et al., 2008; Diem & Lentner, 1973; Mills, Jenkins, Alcocer, & Shewry, 2004). Abdel-Aal (2008) indicated that the choice of proteolytic enzymes, digestion conditions, and the method used for analysis of protein hydrolysates have considerable impact on protein digestibility. They found that the three-enzyme (trypsin, chymotrypsin, and peptidase) one-step digestion gave approximately 39–66% higher protein digestibility than that obtained by the two-enzyme (pepsin and pancreatin) two-step digestion method depending on the type of product and the method used for determining protein hydrolysates. Therefore, Abdel-Aal (2008) suggested that the three-enzyme digestion method is more comparable to *in vivo* conditions. Thus, we assume that *in vitro* digestion methods that use complex enzymes (e.g., a mix of saliva, gastric juice, duodenal juice or bile juice) have the advantage of being more reproducible than those that use single enzymes. Fatouros, Bergenstahl, and Mullertz (2007) reported that the levels of bile salt and phospholipids, the composition (triglyceride, surfactants) and particle size of the formulation, and the levels of calcium chloride and pancreatic lipase might have an impact on the formation of intermediate lipolytic products and colloidal phases and, consequently, the overall performance of the formulation. Therefore, enzyme composition and concentrations may be influenced by the characteristics of the sample.

Several studies showed that the number and type of proteolytic enzymes, digestion conditions, and analysis of protein hydrolysates employed in *in vitro* digestion produced different digestibility results (Abdel-Aal, 2008). Boisen and Eggum (1991) reported that increase in dietary protein induces an increased secretion of pancreatic proteolytic enzymes, while an increase in starch or lipid intake induces may increase secretions of amylase and lipase, respectively.

3.3. Amylase

Amylase is present in the mouth and stomach and is mainly responsible for the conversion of starches to oligosaccharides and monosaccharides (glucose). Amylase is routinely used for *in vitro* digestion studies of plant-based food samples. For instance, Koh, Kasapis, Lim, and Foo (2009), Zhang, Abe, Takahashi, and Sasahara (1996), Bravo, Siddhuraju, and Saura-Calixto (1998), and Evans and Thompson (2004) used α -amylase for starch digestion studies, whereas Nunes, Correia, Barros, and Delgadillo (2004) employed pepsin in a sorghum digestion study.

Thus, the most appropriate composition and concentration of enzymes, such as lipase, pepsin, trypsin, and α -amylase, used within an *in vitro* digestion model must be considered for each specific food sample. As mentioned above, several studies (Almaas et al., 2006; Chattertona et al., 2004; Kitabatake & Kinekawa, 1998) have utilised enzymes collected from human subjects. However, several studies have suggested that the replacement of human pancreatic lipase and co-lipase with porcine pancreatic lipase and co-lipase is generally acceptable (Zangenberg et al., 2001). Thus, it may be very difficult to define which enzymes are better for *in vitro* digestion, and more research is needed in order to analyse the advantages and disadvantages of using enzymes from human subjects. Various *in vitro* methods have been developed to predict the digestibility or physiological changes of food samples. However, predicting the bioavailability and digestion from the food matrix is very difficult, as it depends on many factors associated with food composition and structure. Usually, *in vitro* methods are based upon starch digestion by α -amylase, lipid digestion by lipase, and/or protein digestion by pepsin or trypsin. Gastric digestion is imitated using pepsin at pH around 2. The protease precursors – pepsinogens – produced by chief cells of the stomach, are optimally activated at a pH between 1.8 and 3.2 in the gastric lumen (Jensen-Jarolim, 2006). This indicates that any elevation in the pH may result in a limitation of peptic degradation (Jensen-Jarolim, 2006). Bile salt did not inhibit the lipolytic activity at pH 5.5. Moreover, the changes in the pH in the stomach and intestine can be influenced by the initial pH or amount of the samples tested. Thus, pH is also an important factor for *in vitro* digestion systems. Therefore, the choice of enzyme characteristics such as composition, concentration, and pH should be considered according to sample characteristics.

4. *In vitro* digestion and sample conditions

The characteristics of foods, enzyme type, and enzyme concentrations are key factors that control the digestion of foods during *in vitro* digestion. Abdel-Aal (2008) reported that the differences in digestibility reflect influences of proteolytic enzymes, digestion conditions, as well as the status of protein sources (raw versus processed). Increase in dietary protein induces an increased secretion of pancreatic proteolytic enzymes, while an increase in starch or lipid intake induces increased secretions of amylase and lipase, respectively (Boisen & Eggum, 1991). Thus, *in vitro* digestion characteristics such as digestion time, enzyme contents, or enzyme composition must be adjusted according to sample characteristics. For instance, if the concentration of the target substance (protein, lipid, or carbohydrate) is increased, then the concentration of enzymes or the digestion time must be increased even if the rests of the *in vitro* digestion procedure is kept the same. However, Green, Murphy, Schulz, Watkins, and Ferruzzi (2007) reported that the addition of digestive enzymes did not significantly alter the amount of catechin recovered from green tea after passing through an *in vitro* digestion model. They found that the amount of catechin recovered was similar using an *in vitro* digestion model containing digestive enzymes, as had been reported using an approach that used no enzymes (Record & Lane, 2001). This may be because humans (monogastric stomach) cannot digest plant-based foods well, and so the presence or absence of enzymes had little impact on the release of catechin.

5. Digestion and transit time

The digestion time for each step (e.g., mouth, stomach, and small intestine) is an important factor to establish when designing an appropriate *in vitro* digestion model. *In vivo*, the digestion time depends upon individual characteristics (age, sex, health status,

mental state, time of day) and food properties (total amount, composition, particle size), and may vary quite considerably (McClements et al., 2009). A short transit time of a food within the small intestine may limit the absorption of bioactive lipophilic compounds, thereby reducing their bioavailability (Dahan & Hoffman, 2008). Van Citters and Lin (1999) reported that lipids in the gastrointestinal tract delay the gastric emptying, i.e., the gastric transit time is increased. Therefore, in the case of testing high-lipid food samples, enzymes (lipase or pancreatin) and bile salt/phospholipid amounts and digestion time should be increased in an *in vitro* digestion system. *In vitro* digestion models do not usually take the large intestine into account, because the absorption of compounds mainly takes place mainly in the small intestine (Brandon et al., 2006). Therefore, Brandon et al. (2006) reported that only the bioaccessibility determined in the chyme of the small intestine is relevant for risk assessment. In general, lipids cannot be fermented; thus, lipids are less influenced during passage through the large intestine. Thus, the transit time or digestion time should be shorter in lipid-based food samples than in plant-based food samples in *in vitro* digestion models. In this survey, a digestion time (the stomach, small intestine and large intestine each) of 2 h was used in many *in vitro* digestion models. However, the transit time or digestion time must be considered according to the food characteristics.

6. *In vitro*–*in vivo* Correlation

In vitro–*in vivo* correlations in digestion models are extremely important (Fatouros & Mullertz, 2008). A few studies have evaluated the *in vitro*–*in vivo* correlation of food samples. In an early study, Reymond and Sucker (1988) reported that only a limited amount of digestion had occurred for long chain triglycerides after 12 min of *in vitro* digestion, with the remaining undigested oil retaining the majority of the drug molecules (Reymond & Sucker, 1988). However, Dahan and Hoffman (2007) reported that a trend similar to that obtained in the *in vitro* lipolysis model was observed in *in vivo* experiments for lipophilic drug samples. Brandon et al. (2006) reported that the *in vitro* digestion models developed for food and soil could be partially validated by comparing the bioaccessibility with human *in vivo* bioavailability data or with animal data. Validation of the developed *in vitro* digestion models for consumer products is difficult, because human *in vivo* data from consumer products with contaminants are scarce (Brandon et al., 2006). Fatouros and Mullertz (2008) reported that the *in vitro* solubilisation data correlated well with the *in vivo* data for lipid-based drug samples. However, several studies (Armand et al., 1992; Armand et al., 1997; Marciani et al., 2007) showed that *in vivo* feeding studies demonstrated large differences in the microstructure of emulsions as they pass through the gastrointestinal tract depending on emulsifier type.

7. Conclusions

The present study details various *in vitro* digestion systems, food samples, and measurement parameters. Several researchers have used *in vitro* digestion methods to analyse structural changes, bioavailability, and digestibility of foods, indicating that *in vitro* digestion systems are common and useful tools for analyses of foods and drugs. However, several differences are observed between *in vitro* models and *in vivo* studies. Indeed, *in vitro*–*in vivo* correlations are very important factors. There is clearly an urgent need for more research into *in vitro*–*in vivo* correlations with well-defined systems, so that more realistic *in vitro* models can be developed to screen the bioavailability and digestibility of foods. Moreover, further research is needed to analyse the advan-

tages and disadvantages of *in vitro* digestion models for different food samples.

Acknowledgements

This study was supported by National Research Foundation of Korea Grant funded by the Korean Government (KRF-2009-353-F00006).

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